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**EXHIBIT 2**

# Treatment of Established Tumors with a Novel Vaccine That Enhances Major Histocompatibility Class II Presentation of Tumor Antigen<sup>1</sup>

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## Abstract

Presentation of antigenic peptides by MHC class II molecules to CD4<sup>+</sup> T cells is critical to the generation of antitumor immunity. In an attempt to enhance MHC class II antigen processing, we linked the sorting signals of the lysosome-associated membrane protein (LAMP-1) to the cytoplasmic/nuclear human papilloma virus (HPV-16) E7 antigen, creating a chimera (Sig/E7/LAMP-1). Previously, we found that expression of this chimera *in vitro* and *in vivo* with a recombinant vaccinia vector targeted E7 to endosomal and lysosomal compartments and enhanced MHC class II presentation to CD4<sup>+</sup> T cells compared to vaccinia expressing wild-type E7. In the current study, we tested these recombinant vaccinia for *in vivo* protection against an E7<sup>+</sup> tumor, TC-1, which was derived from primary epithelial cells of C57BL/6 mice cotransformed with HPV-16 E6 and E7 and c-Ha-ras oncogenes. All mice vaccinated with 1 × 10<sup>7</sup> plaque-forming units of wild-type E7-vaccinia showed progressive tumor growth when challenged with a tumorigenic dose of TC-1 tumor cells; in contrast, 80% of mice vaccinated with the chimeric Sig/E7/LAMP1 vaccinia remained tumor free 3 months after tumor injection. Furthermore, treatment with the Sig/E7/LAMP-1 vaccinia vaccine cured mice with small established TC-1 tumors, whereas the wild-type E7-vaccinia showed no effect on this established tumor burden. These findings point out the therapeutic limitations of recombinant vaccinia expressing unmodified tumor antigens. Further, they demonstrate that modifications that reroute a cytosolic tumor antigen to the endosomal/lysosomal compartment can profoundly improve the *in vivo* therapeutic potency of recombinant vaccines.

## Introduction

It is becoming increasingly clear that CD4<sup>+</sup> T cells are critical to the generation of potent antitumor immune responses. CD4<sup>+</sup> T cells have been shown to be instrumental in generating immune responses against several solid malignancies in murine (1, 2) and human systems (3, 4). For example, several mouse tumors transfected with syngeneic MHC class II genes were effective vaccines against subsequent challenge with wild-type, MHC class II-negative tumors (5-7). In an adoptive transfer model, Greenberg *et al.* (8) demonstrated that CD4<sup>+</sup> cells were critical in eliminating FBL tumors in mice. In addition, as crucial memory cells in the T-cell arm of the immune system, CD4<sup>+</sup> cells may provide long-term immunity against specific tumor antigens (9, 10).

CD4<sup>+</sup> T cells recognize antigen in the context of MHC class II molecules. In general, exogenous antigens are taken up by professional antigen-presenting cells through phagocytosis or endocytosis

and are degraded into antigenic peptides by acid proteases in low pH endosomal or lysosome-like compartments (11-13). The antigenic peptides then bind MHC class II molecules and are presented on the cell surface to CD4<sup>+</sup>, MHC class II-restricted T cells. On the other hand, cytoplasmic or nuclear proteins are generally processed and presented to CD8<sup>+</sup> T cells through the MHC class I pathway. Cytoplasmic or nuclear proteins are degraded into peptides in the cytoplasm. The peptides are transported into the endoplasmic reticulum and complexed with MHC class I molecules, which present the antigenic peptides to CD8<sup>+</sup> MHC class I-restricted T cells (reviewed in Ref. 14).

We previously described a novel molecular approach that directly routed a nuclear/cytoplasmic antigen, HPV-16 E7, into the endosomal and lysosomal compartments and enhanced the presentation of antigen to MHC class II-restricted CD4<sup>+</sup> T cells (15). We then constructed a recombinant vaccinia virus containing the chimeric gene, *Sig/E7/LAMP-1*, in which E7 was linked to the endoplasmic reticulum translocation signal peptide, transmembrane domain, and lysosomal targeting domain of LAMP-1 (16-18). LAMP-1 is a type 1 transmembrane protein localized predominantly to lysosomes and late endosomes (19, 20). The cytoplasmic domain of LAMP-1 protein contains the amino acid sequence, Tyr-Gln-Thr-Ile, that mediates the targeting of LAMP-1 into the endosomal and lysosomal compartments (21, 22).

We chose the HPV-16 E7 as a model antigen for the following reasons: (a) HPVs, particularly HPV-16, are associated with most cervical cancers, and the HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and are coexpressed in most HPV-containing cervical cancers. Therefore, vaccines or immunotherapies targeting E7 and/or E6 proteins may provide an opportunity to prevent and treat HPV-associated cervical malignancies; (b) HPV-16 E7 is a characterized cytoplasmic/nuclear protein and is more abundant than E6 in HPV-associated cancer cells; and (c) there were more immunologic assays performed on HPV-16 E7 compared to those on other HPV viral proteins. Therefore, more information is available on E7, and it potentially might be useful for designing immunological assays to study the E7-specific antitumor immune responses (reviewed in Ref. 23).

This specific targeting of HPV-16 E7 to the endosomal and lysosomal compartments allows antigenic peptides of E7 to complex with MHC class II molecules and enhances MHC class II presentation. Specifically, we showed that the *Sig/E7/LAMP-1* vaccinia *in vivo* generated greater E7-specific antibody production and CD4<sup>+</sup> T cell-mediated lymphoproliferative responses than vaccinia expressing the wild-type HPV-16 E7 gene (15). In addition, E7-specific CTL responses were augmented as well, possibly as a consequence of enhanced CD4<sup>+</sup> T-cell help (15).

To determine whether this MHC class II targeting strategy results

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<sup>3</sup> The abbreviations used are: HPV, human papillomavirus; LAMP, lysosome-associated membrane protein; PFU, plaque-forming unit; MAb, monoclonal antibody.

directly in enhanced systemic antitumor responses, we generated an E7-expressing tumorigenic cell line, TC-1. Primary lung epithelial cells from C57BL/6 mice were immortalized by HPV-16 E6 and E7 and then transformed with an activated *ras* oncogene. The cotransformation produced a tumorigenic cell line expressing E6 and E7. This cell line mimics the natural sequence of tumor progression of cervical cancer in which HPV-16 E6 and E7 immortalizes cells and additional mutations transform the cells into advanced tumor cells with metastatic potential. This line thus provides an excellent model to compare the therapeutic potential of recombinant vaccinia expressing the wild-type versus the MHC class II-targeted forms of E7.

## Materials and Methods

**Construction of Mouse Tumor Cells by Cotransformation of HPV-16 E6 and E7 and Activated *ras* Oncogene.** C57BL/6 mouse lungs were dispersed into a single-cell suspension by mechanical grinding, followed by digestion with collagenase at a concentration of 1 mg/ml in DMEM (GIBCO-BRL, Gaithersburg, MD). The primary lung cells were cultured *in vitro* in RPMI 1640, supplemented with 10% fetal calf serum, 50 units/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 2 mM nonessential amino acids, and grown at 37°C with 5% CO<sub>2</sub>. Transduction of HPV-16 E6 and E7 genes into primary lung cells was performed with the LXSNI6E6E7 retroviral vector, a generous gift from Denise A. Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA) (24). The HPV-16 E6 and E7 containing LXSNI6E6E7 were used to infect CRIP cells to generate recombinant virus with a wide host range. The primary lung cells were immortalized by transduction as described previously (24). Following transduction, the retroviral supernatant was removed, and the cells were grown in G418 (0.4 mg/ml) culture medium for an additional 3 days to allow for integration and expression of the recombinant retroviral genes. The immortalized lung (E6+E7) cells were then transduced with pVEJB expressing activated human c-Ha-*ras* gene, kindly provided by Chi V. Dang (The Johns Hopkins Hospital, Baltimore, MD), and selected with G418 (0.4 mg/ml) and hygromycin (0.2 mg/ml). The presence of HPV-16 E7 was confirmed by PCR and immunofluorescent staining.

**Tumor Growth Experiments.** TC-1 tumor cells were injected in five C57BL/6 mice s.c. in the left leg at various doses, including  $1 \times 10^4$ ,  $1 \times 10^5$ , and  $1 \times 10^6$  cells/mouse. Mice were monitored regularly for tumor growth. The data were used to plot a graph of tumor growth kinetics. The animals were then sacrificed, and the tumor nodules were processed to check the presence of HPV-16 E7.

**PCR of HPV-16 E7.** PCRs were performed to determine the presence of E7 DNA in the tumor cells. The primers for HPV-16 E7 open reading frame were based on the published sequence of HPV-16 (25). The 5' primer containing 551–570 bp of HPV-16 sequence was 5'-CCCAGATCTAATCATG-CATG-3', and the 3' primer containing 840–859 bp of HPV-16 sequence was 5'-TATGGTCTGAGAACAGAT-3'. The 100- $\mu$ l reactions containing 1  $\mu$ g of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 6 mM MgCl<sub>2</sub>, 0.5  $\mu$ M of the E7 primers, 0.2 mM each of dATP, dCTP, dTTP, and dGTP, and 2.5 units of ampli-Taq polymerase (Perkin-Elmer Cetus; Norwalk, CT) were subjected to 30 amplification cycles of 1 min of denaturation at 94°C, 1 min of primer annealing at 52°C, and 1 min of extension at 72°C with a thermal cycler (Perkin-Elmer Cetus). Aliquots of reaction products were size fractionated by 2% agarose gel electrophoresis.

**Immunofluorescent Detection of HPV-16 E7 in TC-1 Tumor Cells.** TC-1 cells in tissue culture were centrifuged onto a slide by cytospin, fixed with 4% paraformaldehyde, and permeabilized with 0.1% saponin. Explanted tumor cells were prepared from frozen sections. For the detection of the HPV-16 E7 protein, mouse anti-HPV-16 E7 MAb (Triton Corp., Alameda, CA) was used in conjunction with Texas Red-conjugated goat antimouse IgG (Calbiochem Corp., La Jolla, CA).

**In Vivo Tumor Protection Experiments.** For vaccination,  $1 \times 10^7$  PFUs of each vaccinia (including wild-type vaccinia, E7-vaccinia, and Sig/E7/LAMP-1 vaccinia) were injected i.p. into C57BL/6 mice. For challenge,  $2 \times 10^5$  TC-1 cells/mouse were injected s.c. in the left leg 1 month after vaccination. TC-1 tumor cells growing *in vitro* culture were trypsinized, washed three times in serum-free  $1 \times$  HBSS, and injected. Five mice were used

in each group. Mice were monitored twice a week for tumor growth euthanized after the development of tumor.

**In Vivo Tumor Regression Experiments.** Tumor cells for injection and the vaccinia for immunization were prepared as described above. Three  $\times 10^5$  TC-1 cells were injected s.c. in the left leg. After 7 days,  $1 \times 10^7$  PFU of recombinant vaccinia, either wild-type vaccinia, E7-vaccinia, or Sig/E7/LAMP-1-vaccinia, were injected i.p. into each C57BL/6 mouse. Five mice were used for each vaccinia. Mice were monitored twice weekly and euthanized after the development of tumor.

**In Vivo Antibody Depletion Experiments.** *In vivo* antibody depletions have been described previously (26). Briefly, C57BL/6 mice were vaccinated with Sig/E7/LAMP-1 vaccinia at  $1 \times 10^7$  PFU/mouse and challenged with TC-1 tumor cells at  $2 \times 10^5$  cells/mouse 1 month later. Depletions were started 1 week prior to tumor inoculation. Five C57BL/6 mice were used in each group. MAb GK1.5 (27) was used for CD4 depletions, MAb 2.43 (28) was used for CD8 depletions, and MAb PK136 (29) was used for NK1.1 depletion. Depletion of lymphocyte subsets was assessed on the day of live tumor injection and weekly thereafter by flow cytometric analysis of spleen cells stained with 2.43 or GK1.5. For each time point of analysis, >99% depletion of the appropriate subset was achieved with normal levels of the other subsets. Depletion was terminated on day 45 after tumor inoculation.

## Results

**Creation of a Model Tumor Which Requires E<sub>6</sub> and E<sub>7</sub> For Malignant Phenotypes.** Primary lung cells of C57BL/6 mice were immortalized with HPV-16 E6 and E7 genes and then transformed with pVEJB-expressing activated human c-Ha-*ras* gene. The cotransformation produced a tumorigenic cell line expressing E6 and E7. Morphologically, TC-1 tumor cells showed pleomorphic nuclear patterns, high mitotic index, abnormal mitosis, and high nuclear:cytoplasmic ratio, all commonly seen in malignant neoplasms (Fig. 1A). Immunocytochemical staining for cytokeratin, which is characteristic of epithelial-derived tumors, was positive for TC-1 (data not shown). In addition, the presence of E7 DNA was confirmed by PCR (Fig. 1B), and E7 proteins were detected by immunofluorescent staining (Fig. 1C). TC-1 is MHC class I<sup>+</sup> and MHC class II<sup>-</sup> (data not shown). Tumor growth kinetics were determined by injecting various doses of TC-1 cells into C57BL/6 mice. All the mice injected with TC-1 cells at  $1 \times 10^6$  or  $1 \times 10^5$  cells/mouse had tumor growth; at a dose of  $1 \times 10^4$  cell/mouse, 80% (4 of 5) of mice injected with TC-1 tumor cells developed tumors (Fig. 2).

**Vaccination with Sig/E7/LAMP-1 Vaccinia but not E7 Vaccinia Generates Systemic Responses against Challenge with the TC-1 Tumor.** C57BL/6 mice were immunized i.p. with  $1 \times 10^7$  PFU of either wild-type, E7, or Sig/E7/LAMP-1 vaccinia; 1 month later, the mice were challenged s.c. with  $2 \times 10^5$  TC-1 cells. As shown in Fig. 3, 80% of mice receiving Sig/E7/LAMP-1 vaccinia remained tumor-free 3 months after tumor injection, while those injected with wild-type vaccinia or E7-vaccinia developed tumors within 3 weeks. Vaccination with E7-vaccinia slightly delayed tumor growth relative to wild-type vaccinia, but the effect was minimal. To assess treatment of established tumors, TC-1 cells were first injected into C57BL/6 mice s.c. at a dose of  $3 \times 10^4$  cells/mouse in the left leg. Seven days later, these mice also received  $1 \times 10^7$  PFU of either wild-type vaccinia, E7-vaccinia, or Sig/E7/LAMP-1-vaccinia i.p. As shown in Fig. 4, only those receiving Sig/E7/LAMP-1 vaccinia remained tumor free; all of the mice injected with wild-type vaccinia or E7-vaccinia showed progressive tumor growth within 2 weeks after tumor inoculation. Tumors that grew in mice vaccinated with E7-vaccinia still expressed E7 by PCR and immunofluorescence analysis (data not shown), indicating that the low therapeutic efficacy was not due to antigen loss.

**CD4<sup>+</sup>, CD8<sup>+</sup> T Cells, and NK1.1 Cells Were Essential for the Antitumor Immune Response Generated by Vaccination with Vaccinia Sig/E7/LAMP-1.** To determine the types of lymphocytes that are important for the rejection of E7-positive tumor cells in our *in*

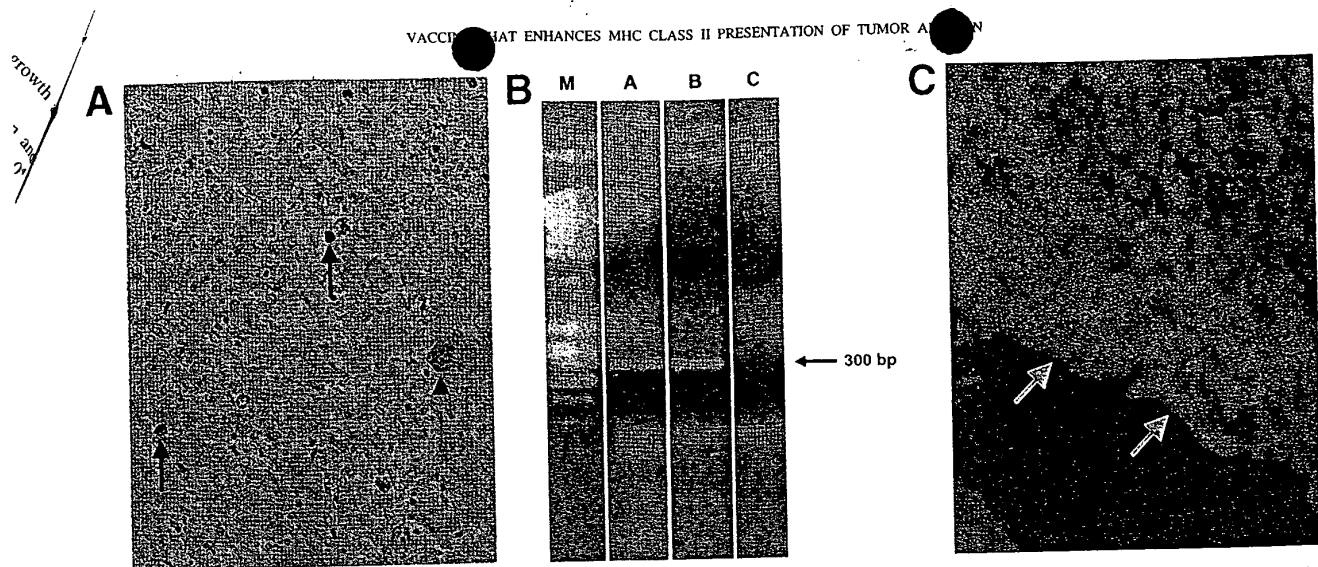


Fig. 1. Generation and characterization of E7-expressing TC-1 tumor. Tumors were explanted from C57BL/6 mice s.c. injected with TC-1 cells. A, hematoxylin and eosin staining of the explanted tumor. The tumor displays a high level of mitotic activity, pleomorphic nuclei (arrowhead), abnormal mitoses (arrows), and high nuclear:cytoplasmic ratio.  $\times 250$ . B, demonstration of the presence of E7 DNA within the genome of cells of explanted tumors by PCR and gel electrophoresis. Lane M, size marker. Lane A, positive control using DNA from CaSki cells, an HPV-16-containing cell line. Lane B, DNA from explanted TC-1 tumor. Lane C, negative control using HPV-negative human placenta DNA. A 300-bp DNA fragment corresponding to the size of the HPV-16 E7 open reading frame was observed in Lanes A and B as indicated by the arrow. C, immunofluorescent staining to demonstrate the expression of HPV-16 E7 protein in the explanted TC-1 tumor. Tumor sections were fixed, permeabilized, and stained with anti-HPV-16 E7 mouse MAb, followed by Texas red-conjugated goat antimouse IgG secondary antibodies. Arrows, the junction between infiltrating tumor and surrounding normal tissue. Diffuse cytoplasmic and nuclear staining were noted in the positive cells but not in the surrounding stromal tissues.  $\times 250$ .

*in vivo* model, we performed *in vivo* antibody depletion experiments. Depletion of lymphocyte subsets was assessed on the day of tumor injection and weekly thereafter by flow cytometric analysis of spleen cells. More than 99% depletion of the appropriate subset was achieved with normal levels of the other subsets (data not shown). As shown in Fig. 5, all mice depleted of CD4<sup>+</sup>, CD8<sup>+</sup> T cells or NK1.1 cells grew TC-1 tumors, in contrast to nondepleted mice which remained tumor free. Mice depleted of either CD4<sup>+</sup> or CD8<sup>+</sup> T cells grew tumors 2–3 weeks after injection; those depleted of NK1.1 cells grew tumors 4–5

weeks after tumor injections. These results suggest that CD4<sup>+</sup>, CD8<sup>+</sup> T cells and NK1.1 cells are essential for the antitumor immunity generated by Sig/E7/LAMP-1 vaccinia.

#### Discussion

In this study, we have demonstrated that Sig/E7/LAMP-1 vaccinia induced potent E7-specific antitumor immunity. Vaccination with vaccinia Sig/E7/LAMP-1 protected mice from challenge and elimi-

Fig. 2. Tumor growth kinetics of TC-1. TC-1 tumor cells were injected into C57 BL/6 mice s.c. at various doses ( $1 \times 10^6$ ,  $1 \times 10^5$ , or  $1 \times 10^4$  cells/mouse). The mice were monitored for evidence of tumor growth by palpation and inspection twice a week.

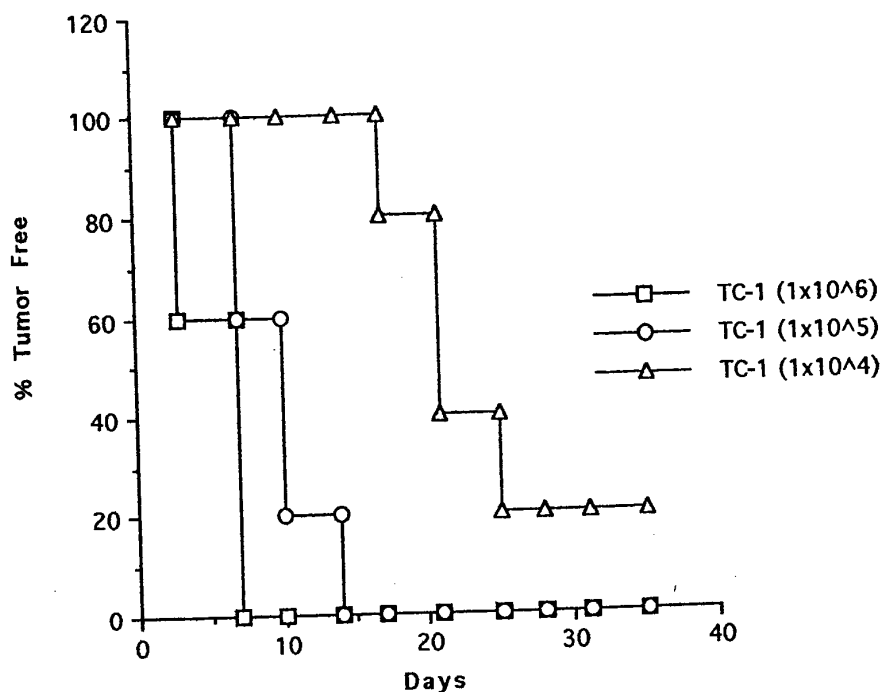
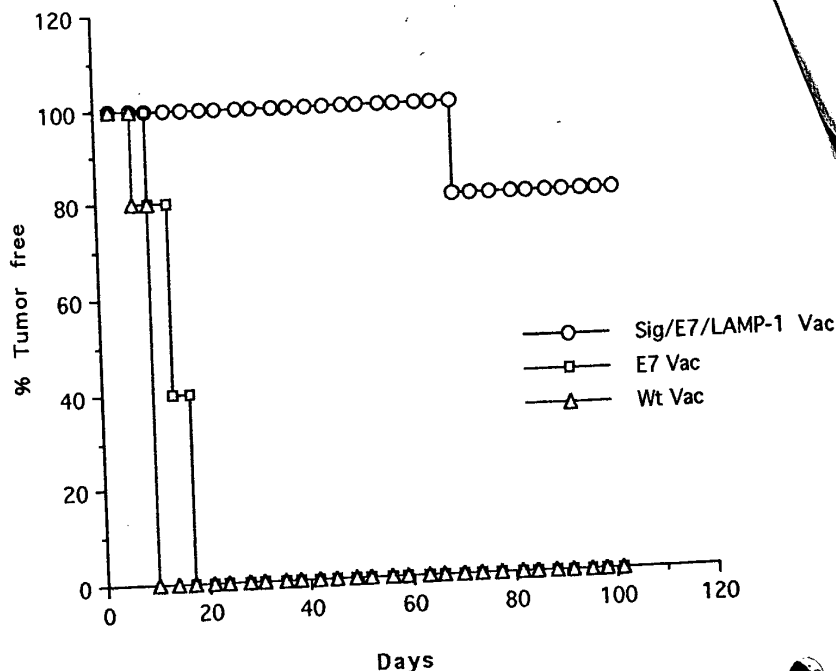


Fig. 3. Sig/E7/LAMP-1 vaccinia protects mice against challenge with TC-1 tumor. C57BL/6 mice were either not immunized or were immunized i.p. with wild-type (nonrecombinant) vaccinia, E7-vaccinia, or Sig/E7/LAMP-1-vaccinia at  $1 \times 10^7$  PFUs/mouse. A month later, mice were challenged s.c. with TC-1 tumor at  $2 \times 10^5$  cells/mouse. Tumor growth was assessed as in Fig. 2.



nated established E7-expressing tumors. We have demonstrated previously that Sig/E7/LAMP-1 vaccinia is capable of enhancing both CTL activity and helper T-cell proliferation (15). Thus, our results suggested that the potent antitumor immunity is correlated with the enhanced CD8<sup>+</sup> and CD4<sup>+</sup> T-cell responses generated by Sig/E7/LAMP-1 vaccinia and that both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are important in the antigen-specific, antitumor immune responses. The *in vivo* antibody depletion studies also demonstrated the requirement of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells for the generation of potent antitumor

immunity. Surprisingly, NK 1.1 cells were also found to be important in the antitumor immunity generated by Sig/E7/LAMP-1 vaccinia. We have demonstrated previously the corequirement for CD4<sup>+</sup> T cells and NK cells in the immune response to tumors that are negative for MHC class I expression (26). In those studies, the depletion of NK cells resulted in the selective outgrowth of tumors that have down-regulated MHC class I expression.

Tumor growth in the vaccinated mice was not due to a consequence of antigen loss since E7 DNA and protein were present in the ex-

Fig. 4. Sig/E7/LAMP-1 vaccinia eliminates established TC-1 tumor. C57BL/6 mice were injected s.c. with TC-1 tumor at  $3 \times 10^4$  cells/mouse and received either no treatment or immunization with wild-type vaccinia, E7-vaccinia, or Sig/E7/LAMP-1-vaccinia i.p. at  $1 \times 10^7$  PFUs/mouse 7 days later. Tumor growth was assessed as in Fig. 2.

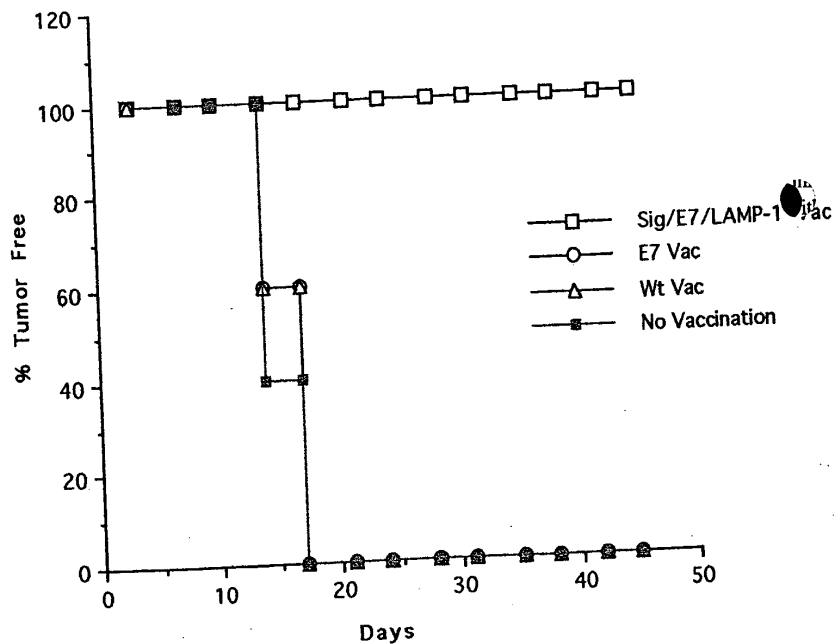
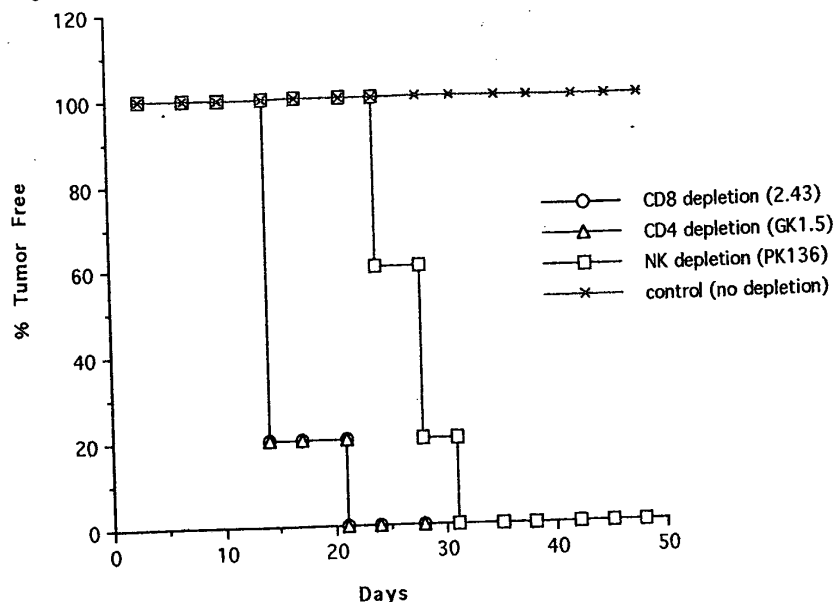


Fig. 5. Effect of lymphocyte subset depletions on the potency of Sig/E7/LAMP-1 vaccinia. C57BL/6 mice were immunized i.p. with Sig/E7/LAMP-1 vaccinia at  $1 \times 10^7$  PFUs/mouse and challenged s.c. with TC-1 tumor at  $2 \times 10^5$  cells/mouse 1 month later. Depletions were started 1 week prior to tumor inoculation. MAb GK1.5 (27) was used for CD4<sup>+</sup> T-cell depletion ( $\Delta$ ), MAb 2.43 (28) was used for CD8<sup>+</sup> T-cell depletion ( $\circ$ ), and MAb PK136 (29) was used for NK1.1 cell depletion ( $\square$ ). Mice without lymphocyte subset depletions were used as control ( $\times$ ). Tumor growth was assessed as in Fig. 2.



planted TC-1 tumor cells. The ability of tumors to lose specific immunogenic antigens must be considered while evaluating an antigen-specific immunotherapy. An appropriate tumor model for HPV-associated cervical cancer should carry the E6 and E7 antigens in a form that is also required for the maintenance of its malignant phenotype; these tumor cells cannot avoid immune responses directed at the antigen by turning off or deleting the antigen since such cells would cease to be tumorigenic.

The new TC-1 tumor cell line was developed to circumvent the confounding issue of negatively selecting nonessential tumor markers in cancer vaccine experiments. Primary lung epithelial cells from C57BL/6 mice were immortalized with HPV-16 E6 and E7 and then transformed with the activated *ras* oncogene. This transformation process mimics the natural sequence in the pathogenesis of cervical cancer in which HPV-16 plays a critical role in carcinogenesis. Since E6 and E7 are required for the induction and maintenance of the malignant phenotype (30), the TC-1 tumor cells cannot avoid E7- and E6-directed immune recognition by losing E6 or E7 while remaining malignant.

As striking as the therapeutic effect of Sig/E7/LAMP-1 vaccinia is the lack of efficacy of E7-vaccinia. Our studies, therefore, suggest that modifications of recombinant poxvirus vaccines that enhance targeting of antigens into MHC processing pathways may greatly improve their clinical utility. Meneguzzi *et al.* reported protection by recombinant vaccinia expressing E6 and E7 in a different E6 and E7 expressing rat tumor but did not report any results on treatment of established tumors (31). It is possible that the combined immune responses targeted at both E6 and E7 resulted in more effective protection than we observed using only E7-vaccinia. Alternatively, the tumor used in those studies may have been more immunogenic or intrinsically less tumorigenic than TC-1.

In summary, we have demonstrated that the Sig/E7/LAMP-1 recombinant vaccinia can generate strong E7-specific antitumor immunity and that specific intracellular antigen targeting strategies might be successfully used to enhance the presentation of tumor-specific antigens, thereby increasing antigen-specific antitumor immune responses.

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